DRUG SUSCEPTIBILITY TESTING OF *Mycobacterium tuberculosis* USING DIRECT TETRAZOLIUM MICROPLATE ASSAY (TEMA)

MOHAMMAD LUKMAN BIN YAHAYA

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by

MOHAMMAD LUKMAN BIN YAHAYA

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DEDICATION

I dedicate my thesis work to my family and many friends. A special feeling of gratitude to my loving parents, Yahaya Bin Ishak and Asmah Binti Abdullah whose words of encouragement and push for tenacity ring in my ears.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degree Celsius
µg	Microgram
µL	Microliter
ACM	Absolute Concentration Method
AFB	Acid Fast Bacilli
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
AUC	Area under Curve
BSC	Bio-safety Cabinet
CFU	Colony Forming Units
CI	Confident Interval
CIP	Ciprofloxacin
CO_2	Carbon Dioxide
CSF	Cerebrospinal Fluid
dH_2O	Distilled Water
DIL	Dilutions
DNA	Deoxyribonucleic Acid
DR-TB	Drug Resistant Tuberculosis
DST	Drug Susceptibility Testing
DTH	Delayed-Type Hypersensitivity
EMB	Ethambutol
FDA	Food and Drug Administration
g	Gram
H37Rv	*Mycobacterium tuberculosis* Wild Strain
HRPZ II	Hospital Raja Perempuan Zainab II
i.e.	That is
IM	Intramuscular
INH	Isoniazid

IPR	Institut Perubatan Respiratori
Lab.	Laboratory
LAMP	Loop-Mediated Isothermal Amplification
LED	Light Emitting Diode
L-J	Lowenstein-Jensen
MDR-TB	Multidrug Resistant Tuberculosis
mg	Miligram
MGIT	Mycobacterial Growth Indicator Tube
MHC	Major Histocompatibility Complex
MIC	Minimum Inhibitory Concentration
MKAK	Makmal Kesihatan Awam Kebangsaan
mL	Milliliter
MODS	Microscopic Observation Drug Susceptibility
MTB	*Mycobacterium tuberculosis*
MTT	Dimethylthiazol-diphenyltetrazolium Bromide
NTB	Non-tuberculosis Mycobacteria
O_2	Oxygen
OADC	Oleic Acid, Dextrose and Catalase
PANTA	Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim and Azlocillin
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PM	Proportion Method
PPD	Purified Protein Derivative
PPSP	Pusat Pengajian Sains Perubatan
PVR	Predictive Value for Resistance
PVS	Predictive Value for Susceptibility
PZA	Pyrazinamide
RCF	Relative Centrifugal Force
RMP	Rifampicin

ROC	Receiver Operating Characteristics
SD	Standard Deviation
SM	Streptomycin
SPSS	Statistical Package for Social Sciences
TAT	Turnaround Time
TB	Tuberculosis
TEMA	Tetrazolium Microplate Assay
TTC	2,3,5-triphenyltetrazolium Chloride
USA	United State of America
USD	United State Dollar
USM	Universiti Sains Malaysia
UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume
WHO	World Health Organization
x g	Standard Acceleration Due to Gravity
XDR-TB	Extensively Drug-Resistant Tuberculosis
Z-N	Ziehl-Neelsen
Δ	Precision

UJIAN KERENTANAN UBAT TERHADAP *Mycobacterium tuberculosis* MENGGUNAKAN ASSAI MIKROPLAT TETRAZOLIUM (TEMA) LANGSUNG

ABSTRAK

Assai yang cepat, murah dan tinggi daya pemprosesan untuk ujian kerentanan ubat *Mycobacterium tuberculosis* (MTB) amat diperlukan terutamanya di negara-negara membangun yang mana kes batuk kering adalah berleluasa. Matlamat kajian ini adalah untuk menilai ujian kerentanan MTB terhadap ubat anti-TB barisan pertama dengan mengggunakan assai mikroplat tetrazolium (TEMA) langsung ke atas spesimen klinikal (kahak) tanpa keperluan untuk pemencilan MTB terlebih dahulu seperti yang biasanya dilakukan dalam TEMA tidak langsung. Sebanyak 59 sapuan spesimen kahak positif (AFB) telah dimasukkan secara langsung ke dalam kaldu media 7H9-S bebas ubat dan yang mengandungi pencairan bersiri ubat menggunakan pewarna tetrazolium sebagai petunjuk pertumbuhan dalam mikroplat. Semua kategori sapuan spesimen kahak positif (AFB) dengan bilangan berbeza (dari sedikit hingga 3+) telah digunakan dalam TEMA langsung manakala untuk TEMA tidak langsung, saiz inokulum piawai sebanyak 1.50×10^7 CFU/mL telah digunakan. Kepekatan perencat minima (MIC) untuk isoniazid (INH), rifampicin (RMP), ethambutol (EMB) dan streptomycin (SM) telah diperolehi dari TEMA langsung dan tidak langsung dengan merujuk kepada kaedah kepekatan mutlak (ACM). Lengkung (ROC) telah digunakan untuk menentukan nilai titik pemisah MIC. Sensitiviti, spesifisiti, kejituan, nilai ramalan dan juga tempoh keseluruhan pemprosesan untuk mendapatkan keputusan akhir ujian sensitiviti telah dibandingkan. Lebih daripada 70% strain MTB mempunyai taburan MIC antara 0.0156 hingga 0.0313 µg/mL untuk INH; 0.0005 hingga 0.25 µg/mL untuk RMP; 0.5 hingga 2.0 µg/mL untuk EMB dan 0.0625 hingga 0.25 µg/mL untuk SM menggunakan TEMA tidak langsung

manakala untuk TEMA langsung MIC bertaburan antara 0.0039 hingga 0.0625 µg/mL untuk kedua-dua INH dan RMP; 0.25 hingga 1.0 µg/mL untuk EMB dan 0.0625 hingga 0.25 µg/mL untuk SM. TEMA langsung mempamerkan prestasi yang baik dengan secara tepat membezakan strain MTB yang rintang dan rentan terhadap ubat anti-TB barisan pertama sepertimana yang diperlihatkan oleh luas di bawah lengkungan (ROC) antara 0.7569 hingga 0.9643. Untuk TEMA tidak langsung, sensitiviti yang diperoleh masing-masing untuk INH, RMP, EMB dan SM adalah 80%, 71%, 75% dan 100% manakala spesifisiti adalah 96%, 60%, 38% dan 84% masing-masing untuk INH, RMP, EMB dan SM. Untuk TEMA langsung pula, sensitiviti sebanyak 100% diperoleh untuk INH, EMB dan SM manakala 71% untuk RMP. Sebaliknya, spesifisiti untuk INH, RMP, EMB dan SM masing-masing adalah 80%, 71%, 55% dan 93%. Secara keseluruhannya, kejituan dan nilai ramalan untuk TEMA langsung adalah setanding dengan TEMA tidak langsung. Tempoh pemprosesan keseluruhan 15 hari diperoleh dengan TEMA langsung diikuti dengan TEMA tidak langsung (39 hari) dan ACM (100 hari) ($P < 0.001$). Kesimpulannya, TEMA langsung adalah kaedah yang lebih mudah, cepat dan boleh dipercayai untuk ujian saringan kerentanan ubat MTB di negara-negara yang mempunyai peningkatan kadar prevalen strain ketahanan ubat.

DRUG SUSCEPTIBILITY TESTING OF *Mycobacterium tuberculosis* USING DIRECT TETRAZOLIUM MICROPLATE ASSAY (TEMA)

ABSTRACT

A rapid, inexpensive and high-throughput assay for drug susceptibility testing (DST) of *Mycobacterium tuberculosis* (MTB) is urgently required especially in developing countries where TB cases are prevalent. The aim of this study was to evaluate the drug susceptibility testing (DST) of MTB to the first-line anti-TB drug using tetrazolium microplate assay (direct TEMA) performed directly on clinical specimens (sputum) by omitting the need for prior isolation of MTB in sputum specimens currently performed by indirect TEMA. A total of 59 acid fast bacilli (AFB) smear positive sputum specimens were directly inoculated into drug-free and serially diluted drug in 7H9-S broth media using tetrazolium dye as growth indicator in the microplate wells. All AFB smear categories with different microscopic bacilli counts (from scanty to 3+) were included in the direct TEMA while the standard inoculum size used in the indirect TEMA was 1.50×10^7 CFU/mL. The minimum inhibitory concentrations (MICs) of isoniazid (INH), rifampicin (RMP), ethambutol (EMB) and streptomycin (SM) were obtained for direct and indirect TEMA with reference to the absolute concentration method (ACM). Receiver Operating Characteristics (ROC) curve was used to determine the cut-off MIC values. The sensitivity, specificity, accuracy and predictive values as well as the mean turnaround time (TAT) for the final sensitivity test results were compared. The MIC for more than 70% of MTB strains were distributed between 0.0156 to 0.0313 μg/mL for INH; 0.0005 to 0.25 μg/mL for RMP; 0.5 to 2.0 μg/mL for EMB and 0.0625 to 0.25 μg/mL for SM for indirect TEMA whereas 0.0039 to 0.0625 μg/mL for both INH and RMP; 0.25 to 1.0 μg/mL for EMB and 0.0625 to 0.25 μg/mL for SM for direct TEMA. The

direct TEMA method performed well by accurately distinguishing between the resistant and susceptible strains of MTB as seen by the area under the ROC curve (AUC) ranged from 0.7569 to 0.9643 against the first-line anti-TB drugs. In indirect TEMA, 80%, 71%, 75% and 100% sensitivities were obtained for INH, RMP, EMB and SM respectively while specificities were 96%, 60%, 38% and 84% for INH, RMP, EMB and SM respectively. In the direct TEMA, 100% sensitivity was obtained for INH, EMB and SM and 71% for RMP. However, the specificities for INH, RMP, EMB and SM were 80%, 71%, 55% and 93% respectively. The overall accuracy and predictive values of direct TEMA were comparable to indirect TEMA. A significant shorter mean TAT of 15 days was observed for direct TEMA followed by indirect TEMA (39 days) and ACM (100 days) ($P < 0.001$). In conclusion, direct TEMA is a relatively simple, rapid and reliable method for DST screening of MTB in countries with increasing prevalence rates of drug resistance strains.

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Mycobacterium tuberculosis (MTB) is an infectious agent responsible for tuberculosis (TB) among humans. In recent years TB has emerged as an important public health problem in both developing and developed countries (World Health Organization, 2013). The highest number of TB cases occurs among countries in South-East Asia and Western Pacific Regions which accounted for 56% of cases worldwide in 2013 (World Health Organization, 2014). South-East Asia Region alone accounts for 33% of all cases globally (Vashishtha, 2009). The last ten years evidenced a steady increase in the number of TB cases in Malaysia from about 64 cases per 100 000 population in 2003 (World Health Organization, 2009) to about 72.4 cases per 100 000 population in 2011 (Ministry of Health Malaysia, 2011).

According to 2014 World Health Organization (WHO) Report, the development of multi-drug resistant tuberculosis (MDR-TB) (MTB strain which is resistant to at least the two most powerful anti-TB drugs, INH and RMP) and the emergence of extensively drug resistant tuberculosis (XDR-TB) (MDR-TB that has developed resistance to any member of the fluoroquinolone family and at least one of the three injectable second-line anti-TB drugs: amikacin, kanamycin or capreomycin used to treat MDR-TB) posed a major obstacle in the treatment and control of TB worldwide. An estimated 480 000 MDR-TB cases were reported worldwide with resistance to anti-TB drugs varied from one country to another and in different regions within the same country (Zignol *et al.*, 2006; World Health Organization, 2008; World Health Organization, 2014).

A study done in Malaysia in the late 80's reported that the rate of primary resistance to any type of first-line anti-TB drug was 13 – 15% while multi-drug resistant was about 1% (Jalleh *et al.*, 1993). Another source indicated the prevalence of MDR-TB in Malaysia in 1997 was 0.1% (World Health Organization, 2000). A 2012 statistics has estimated the cases of MDR-TB among notified TB cases in Malaysia at 18 cases (World Health Organization, 2013). Although few studies and statistic put forth to explain the prevalence rate in Malaysia, the systematic data on the prevalence of drug resistant TB (DR-TB) in the Peninsular Malaysia is still not available. The possible reasons for the paucity of information on MTB resistant pattern may be due to the lack of a simple, quick and affordable technique in determining the antibiotic susceptibility in most of the laboratories.

1.2 Rationale of The Study

The availability of a rapid, simple and inexpensive method for drug susceptibility testing (DST) would be very helpful for resources limited laboratories where TB is highly prevalent. Minimum inhibitory concentration (MIC) determination is very crucial in order to monitor susceptibility of MTB as well as to prevent transmission of MDR-TB in the community (Mshana *et al.*, 1998). The present available methods for DST such as the gold standard: absolute concentration method (ACM) and proportion method (PM); automated methods: Radiometric BACTEC 460TB System and BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 System; E-test and new molecular tools seem to be time consuming and expensive for use in the underprivileged settings i.e., low income countries (Franzblau *et al.*, 1998).

Promising results were reported by several studies regarding the simple, low cost and rapid colorimetric assays that could be produced in house for determining

DST of MTB by the use of reagents such as Alamar blue (Yajko *et al.*, 1995; Franzblau *et al.*, 1998), dimethylthiazol-diphenyltetrazolium bromide (MTT) (Gomez-Flores *et al.*, 1995) and 2,3,5-triphenyltetrazolium chloride (TTC) (Mohammadzadeh *et al.*, 2006). The use of TEMA only costs USD 5.04 for each tested strain (Caviedes *et al.*, 2002) compared to BACTEC 12B (USD 6.40) and MGIT (USD 12.00) (Heifets and Cangelosi, 1999). In addition, such colorimetric assays are able to handle multiple samples at a time (Franzblau *et al.*, 1998). Besides, this method can produce high level of specificity and sensitivity in testing the susceptibility of MTB to the first-line anti-TB drugs (Gomez-Flores *et al.*, 1995; Franzblau *et al.*, 1998; Caviedes *et al.*, 2002; Palomino *et al.*, 2002; De Logu *et al.*, 2003; Morcillo *et al.*, 2004; Martin *et al.*, 2005; Mohammadzadeh *et al.*, 2006).

However, the assay described above uses pure culture from the clinical samples. This will not be able to reduce the total turnaround time for the final sensitivity result because it needs initial isolation of the organisms from samples by inoculation onto the Lowenstein-Jensen (L-J) medium for 3 to 4 weeks (Mshana *et al.*, 1998). With that in mind, the colorimetric result could be made available relatively faster by direct inoculation of clinical sample using colorimetric assay for determination of drug susceptibility without initial isolation of MTB.

Cognizant to the above limitation, this study aims to evaluate the performance of tetrazolium microplate assay (TEMA) for the determination of susceptibility of MTB to first-line anti-TB drugs directly from clinical samples (sputum) (direct TEMA) with comparison to the indirect TEMA (using pure culture isolate) and the gold standard method, ACM.

1.3 Objectives of The Study

1.3.1 General Objective

To evaluate the drug susceptibility testing (DST) of MTB to the first-line anti-TB drugs using TEMA performed directly on clinical specimens (sputum).

1.3.2 Specific Objectives

1. To determine the MIC of isoniazid (INH), rifampicin (RMP), ethambutol (EMB) and streptomycin (SM) by indirect TEMA on MTB pure culture isolate.
2. To determine the MIC of INH, RMP, EMB and SM by direct TEMA on AFB smear positive sputum specimens.
3. To determine the sensitivity, specificity, accuracy and predictive values of direct TEMA and indirect TEMA in comparison to the ACM as gold standard.
4. To compare the total turnaround time (TAT) between the direct TEMA, indirect TEMA and ACM.

1.4 Study Framework

Phase 1: Optimization of TEMA

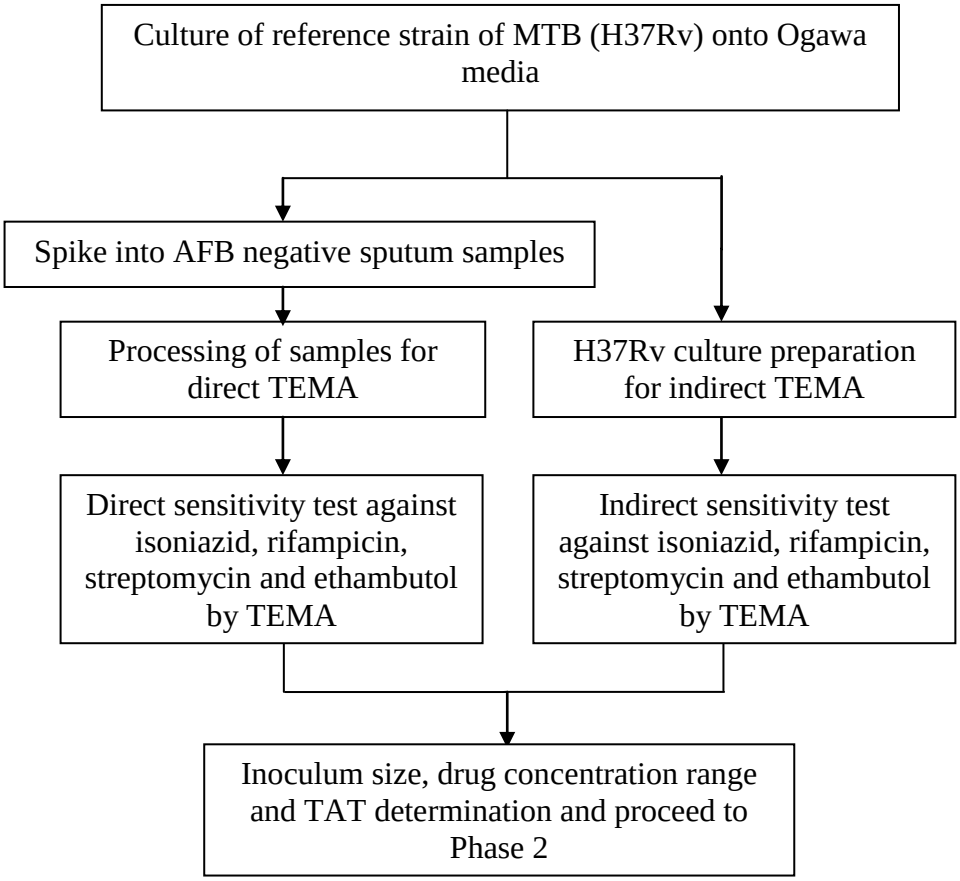


Figure 1.1: The flow chart on the optimization of TEMA

Phase 2: Evaluation of TEMA

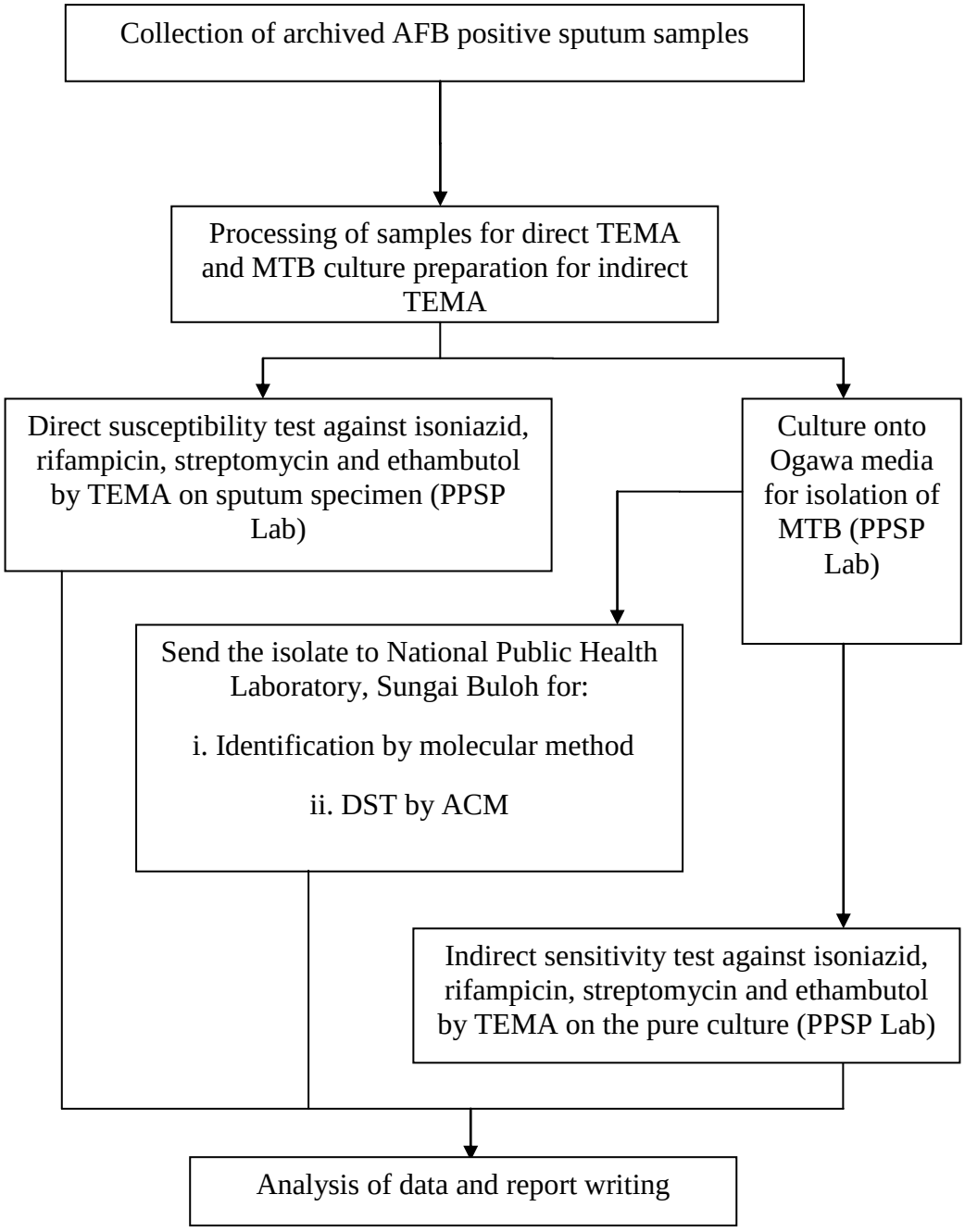


Figure 1.2: The flow chart on the evaluation of TEMA

CHAPTER 2

LITERATURE REVIEW

2.1 *Mycobacterium tuberculosis* (MTB)

The genus *Mycobacterium* comes from the family *Mycobacteriaceae* which is the only genus in this family. MTB and *M. leprae* are two very important human pathogens and respectively cause TB and leprosy whereas the non-tuberculos mycobacteria (NTM) are associated with various kinds of mycobacterioses in humans and animals (Goodwin, 2007; Pfyffer and Palicova, 2011).

Mycobacteria were previously classified based on their phenotypic characteristics which comprise growth rates, photo-reactivity, nutritional and environmental temperature requirements, biochemical test and the range of pathogenicity. Currently, classifications of mycobacteria are done by molecular-based techniques using species-specific rRNA and DNA sequences (Goodwin, 2007; Ryan *et al.*, 2010; Pfyffer and Palicova, 2011).

MTB is slightly curved or straight slim single or branched bacilli, non-motile, 0.2 to 0.6 µm by 1.0 to 10 µm in dimension and possesses acid-fast properties which stained poorly with Gram staining. Its cell wall contains peptidoglycan attached with many branched-chain polysaccharides, proteins and lipids (Goodwin, 2007; Ryan *et al.*, 2010; Pfyffer and Palicova, 2011).

Generally, mycobacteria are aerobic but some species can grow under reduced O_2 atmosphere. MTB grows at optimum temperature of 37ºC and growth enhancement can be observed in the presence of 10% CO_2 and at low pH (6.5 – 6.8). MTB have a slow growth rate and takes about 4 to 6 weeks to observe colonies

growing on solid media. The presence of ammonia or amino acid as nitrogen sources and glycerol as a carbon source in addition to mineral salts are required for the growth of most MTB strains. The growth is also stimulated by fatty acid from egg yolk or oleic acid as well as albumin to neutralize the toxicity of excess fatty acid. The MTB colonies are rough and often appear as prominent patterned texture 'cording' resulting from the tight cohesion of the bacilli (Goodwin, 2007; Ryan *et al.*, 2010; Pfyffer and Palicova, 2011).

Mycobacterial species can be identified by a set of biochemical tests based on the enzymes the organism possesses, metabolic substances and inhibition of growth on exposure to selected biochemical reagents. The set of biochemical tests include niacin accumulation, nitrate reduction, catalase reaction, hydrolysis of Tween-80, iron uptake, arylsulfatase, pyrazinamidase, tellurite reduction and urease test (Goodwin, 2007). Table 2.1 shows biochemical properties of mycobacteria.

MTB complex is commonly isolated from patients suspected with TB. It is characterized by different phenotypes and mammalian host ranges with extreme genetic homogeneity. MTB complex comprises of several mycobacterial species including MTB, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. caprae*, *M. microti*, *M. cenettii* and *M. pinnipedii* (Pfyffer and Palicova, 2011). Table 2.2 describes the details of each member in MTB complex.

Table 2.1: Biochemical properties of mycobacterial species

Test	Organism detected	Interpretation
Niacin accumulation	MTB	Yellow-pigmented compound
Nitrate reduction	MTB, *M. kansasii*, *M. szulgai* and *M. fortuitum*	Forming red colour
Catalase	MTB complex	Heat-stable catalase negative
Hydrolysis of Tween-80	Scotochromogenic mycobacteria	Observing pink color change
Iron uptake	*M. chelonae*	Rusty brown colonies
Arylsulfatase enzyme	Most mycobacterial species	Pink colour
Pyrazinamidase	*M. marinum*, *M. kansasii* and *M. bovis*	Red pigment
Tellurite reduction	*M. avium* complex (MAC)	Colourless to black
Urease	*M. scrofulaceum*	Pink to red

Source: Goodwin (2007)

Table 2.2: Members of MTB complex: source and characteristics

Organisms	Host/Source	Main characteristics
M. tuberculosis	Humans	Off-white and rough in solid media, smoother in moist media
M. bovis	Warm-blooded animals, some prey birds and humans	Small and rounded colonies with irregular edges and granular surface
M. bovis BCG	Vaccine	Predominantly same properties of *M. bovis* except more attenuated in virulence
M. africanum	Humans in tropical Africa	Colonies are the same as MTB in solid media
M. caprae	Goats and cattle	In genomic view, *M. caprae* is identical to the branches of classical *M. bovis*, *M. pinnipedii*, *M. microti* and ancestral MTB but different from modern MTB
M. microti	Guinea pigs, rabbits, llamas, cats and other warm-blooded animals	Appear as 'croissant'-like morphology in stained smears, no growth in culture
M. cenettii	Child and HIV-positive patients	Smooth, round and glossy colonies in solid media
M. pinnipedii	Guinea pigs, rabbits and possible cattle	New member of the MTB complex by host preference, phenotypic and genotypic characteristics

Source: Pfyffer and Palicova (2011)

2.2 Pathogenesis and Clinical Features of Tuberculosis (TB)

TB is an infectious disease that can affect human and animal, where MTB is the major etiologic agent. All members belong to the MTB complex cause TB infections (Ryan *et al.*, 2010).

TB infection begins when airborne droplet nuclei containing MTB enter the respiratory tract by means of inhalation. In the lobes, MTB cells are recognized and phagocytized by alveolar macrophages which undergo lysosomal mechanisms and T-cell response. The T-cell then forms major histocompatibility complex (MHC) molecules. This will lead to cytokine activation (Goodwin, 2007; Ryan *et al.*, 2010; Todar, 2012).

Besides that, MTB multiplication will generate large quantity of MTB protein which can trigger inflammatory elements of delayed-type hypersensitivity (DTH) response (Kobayashi *et al.*, 2001). The DTH with its component causes destruction and injury to cells containing antigen (Goodwin, 2007; Ryan *et al.*, 2010).

Hypersensitivity reaction results in the formation of granuloma. Healing occurs after granuloma formation along with fibrosis, encapsulation and scar formation. Primary infection may heal and the organisms will slowly dying. However, the bacilli may not be totally eradicated but can remain visible for months to years in granulomas and potentially produce reactivation TB later if the cellular immune system of the infected persons is altered or suppressed due to aging, malnutrition, alcoholism, immunosuppressive treatment and AIDS. In this stage, apical or posterior segment of the upper lobe or superior segment of the middle lobe of the lung is commonly involved (Goodwin, 2007; Ryan *et al.*, 2010).

TB infected individual is either asymptomatic or having signs and symptoms of TB disease or may be in extremely debilitated state depending on the stages of infection. Early clinical manifestations are often nonspecific such as malaise and fever. The chest X-ray may show infiltrates in the mid-zones of the lung and also adenopathy, an enlargement of lymph node around the hilum. Ghon's complex characteristic is also present in the X-ray when enlarged lymph nodes fibrosed, combined with calcifications or scars on the middle lobe of lung (Ryan *et al.*, 2010; Swierzewski, 2011).

In active TB, a cough eventually develops in most patients which initially of nonproductive type and later advances to a productive cough with or without blood-stained sputum. Several symptoms such as fever, breathlessness, malaise, fatigue, night sweats, weight loss and signs of pneumonia are also common. The chest X-ray shows the appearance of cavities in lung apices resulting from progressive destruction of lung tissue. The involvement of other organs such as bones, bone marrow, kidneys, lymph nodes, bowel, brain and meninges may occur at this stage. Infected organs manifest with tuberculoma, localized tumor-like mass that is not a cancer and fatal chronic meningitis. Untreated TB patient with progressive cough, fever and weight loss would develop complications such as empyema, pleural fibrosis, massive hemoptysis, adrenal insufficiency, hypercalcemia and if prolong within 2 to 5 years can cause death (Ryan *et al.*, 2010).

2.3 Epidemiology of TB

Humans are the only reservoir of MTB. Most TB infections are transmitted via inhalation of droplet nuclei containing the causative organism. In the situation of poor ventilation, the risk of inhaling the infected droplet nuclei is increasing. Besides

that, TB can also be transmitted through the gastrointestinal tract. It occurs after individual consuming fresh milk directly from TB cattle. However, this mode of transmission is uncommon due to pasteurization of milk (Ryan *et al.*, 2010; Todar, 2012).

2.3.1 Global TB Burden

TB is still a major public health problem worldwide both in developing and developed countries and the second leading cause of death in categories of infectious diseases after the HIV. It has been reported that about 9 million incident cases of TB were estimated worldwide in 2013 (World Health Organization, 2014). Asia contributed the highest number of cases (about 56%), followed by 29% in Africa, 8% in Eastern Mediterranean Region, 4% in European Region and about 3% in the Region of Americas. India contributed the highest numbers of cases with 2.0 – 2.3 million people infected (about 26% of global cases) followed by China (0.9 – 1.1 million cases), Nigeria (340 000 – 880 000 cases), Pakistan (370 000 – 650 000 cases), Indonesia (410 000 – 520 000 cases) and South Africa (410 000 – 520 000 cases) in 2013. About 11 – 14% of TB cases notified in 2013 were among HIV infected patients. The highest incidence rates of TB co-infected with HIV cases were seen in Africa Region in 2013 (World Health Organization, 2014). TB prevalent cases were estimated about 10 – 13 million which equivalent to 159 cases per 100 000 population in 2013. An estimated 1.5 million TB deaths in 2013 among which 73% were HIV-negative and 24% were HIV-positive. Africa and South-East Asia Regions accounted for approximately 78% of total TB deaths globally whereas one-third of these occurred in India and Nigeria alone in 2013 (World Health Organization, 2014). Figure 2.1 shows the estimated percentage of TB incident cases

and mortality rates in 2013 by world region whereas Figure 2.2 shows world map distribution of estimated TB incidence rates in 2013.

2.3.2 TB in Malaysia

Although TB is still a major public health disease in Malaysia, its epidemiological data has not been regularly updated. The rate of TB cases in Malaysia was about 72.4 cases per 100 000 populations in 2011 (Ministry of Health Malaysia, 2011). A total of 15 057 cases of all forms of TB were notified in 2000 (Iyawoo, 2004), increased about 1 – 7% annually until 2010 (Ministry of Health Malaysia, 2010) and in 2011 about 20 666 cases were notified. The state of Sabah accounted for the highest number of TB cases (Dony *et al.*, 2004; Iyawoo, 2004). Factors such as low social-economic and also the high number of immigrant population were associated with increasing TB cases detected in Sabah (Dony *et al.*, 2004). This finding was supported by molecular detection of foreign TB strains among local TB infected patients (Dale *et al.*, 1999). Other factors include rapid urbanization, crowding, dirty environment and malnutrition (Ibrahim, 2010). Among notified cases in 2011, about 61.8% was smear positive cases, 23.5% was smear negative/not known/not done and 14.7% was from extrapulmonary TB cases. There were about 1 644 TB deaths in 2011 giving about 5.8 TB deaths per 100 000 population. Case detection rate and cure rate were 84.1% and 79% respectively in 2011 (Ministry of Health Malaysia, 2011). Figure 2.3 shows decreasing trend of prevalence and incidence rates of TB from 2010 to 2012 in Malaysia. However, the mortality rate was constant throughout the period.

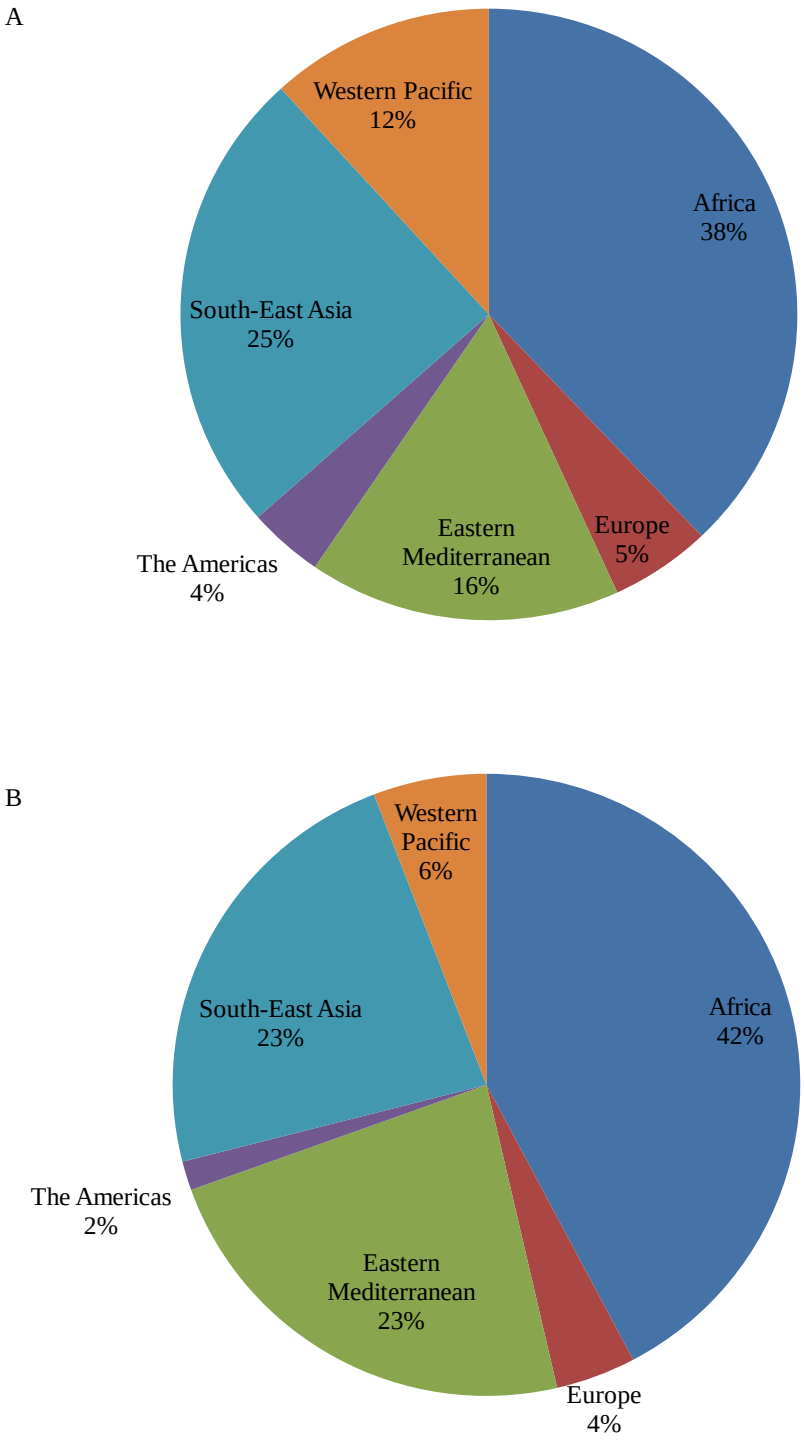


Figure 2.1: World region estimated percentage of A: TB incidence and B: TB mortality rates in 2013 [adapted from World Health Organization (2014)]

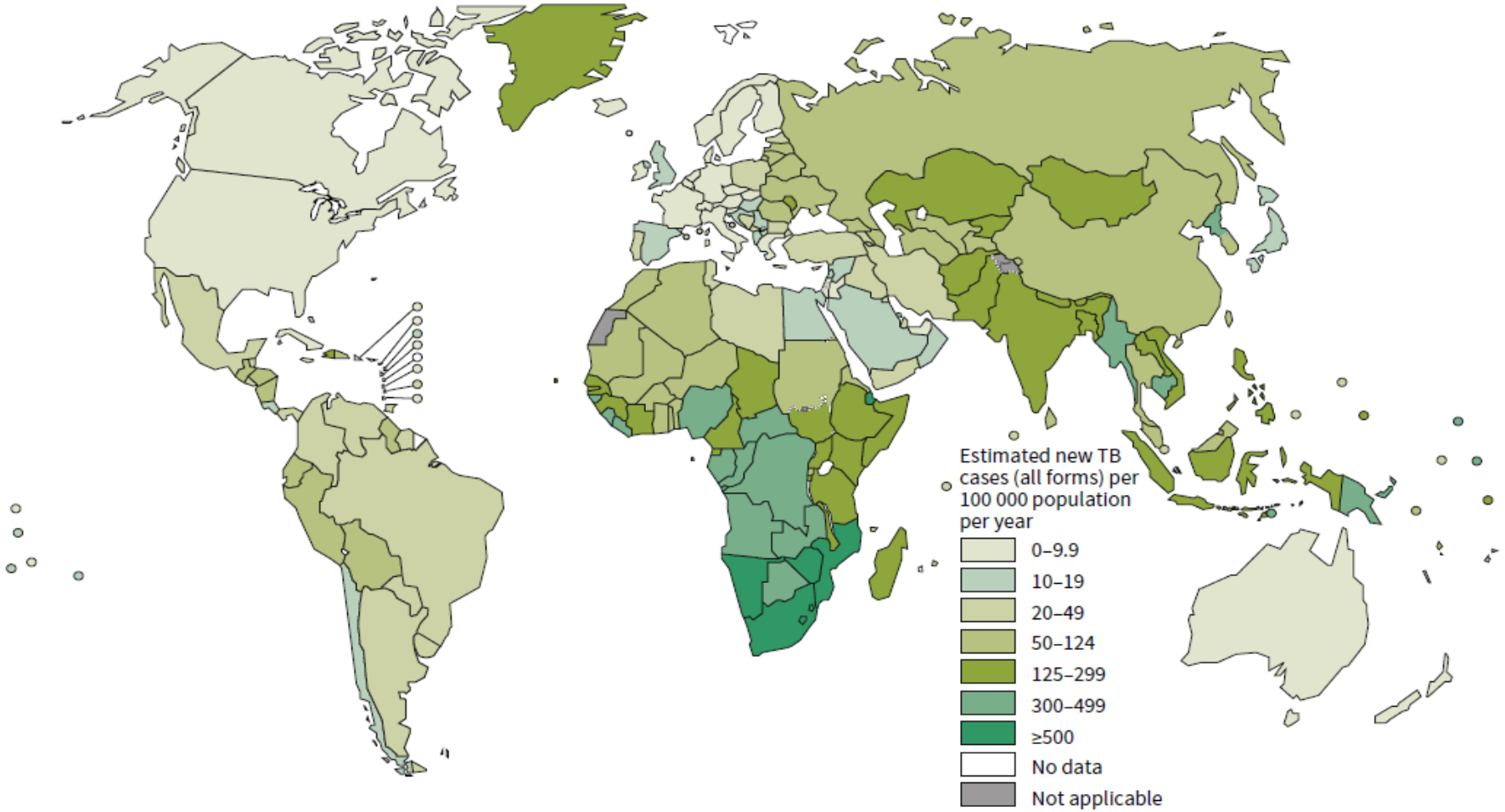


Figure 2.2: World estimated TB incidence rates in 2013 [adapted from World Health Organization (2014)]

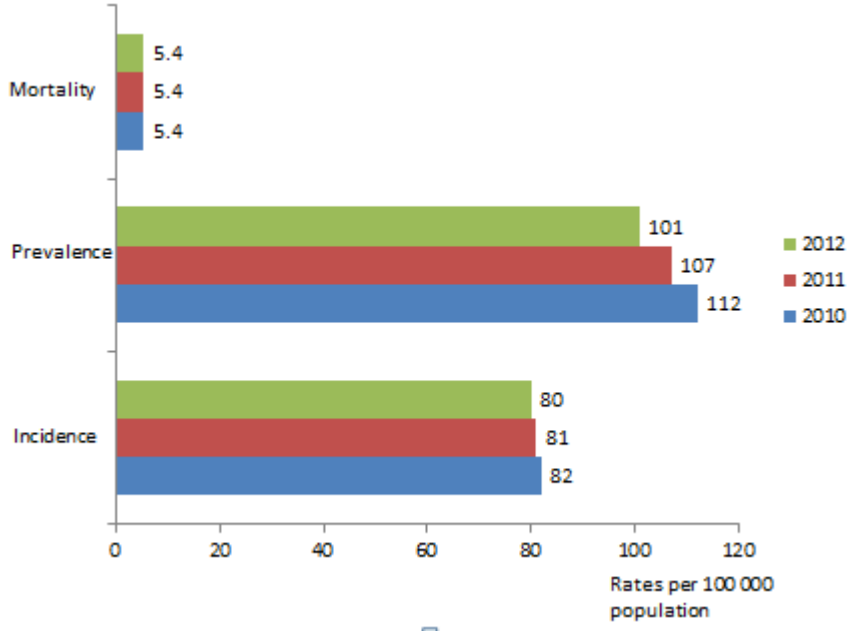


Figure 2.3: Estimated burden of disease caused by TB in Malaysia, 2010 – 2012 [adapted from World Health Organization (2013)]

2.4 Diagnosis of TB

The diagnosis of TB is mainly based on clinical symptoms, radiological finding (chest X-ray) and bacteriological evidence. The laboratory investigations are necessary to diagnose TB and to confirm susceptibility prior to TB drug treatment.

2.4.1 Acid-Fast Stain and Microscopic Examination

Detection of MTB can be performed routinely in laboratory by acid-fast stain method. Stained bacilli are detected microscopically in various clinical specimens such as sputum, cerebrospinal fluid (CSF) and others. There are variations of acid-fast staining techniques. In the Ziehl-Neelsen stain (Z-N stain) procedure, AFB is detected as bright red against a blue background. Other method is the fluorescent acid-fast stain where AFB appears as luminescent yellow-green against a dark background (Ryan *et al.*, 2010; Talaro and Chess, 2011). Recently there are recommendations to use the fluorescent light emitting diode (LED) microscope which gives more advantages (Wilson, 2011).

2.4.2 Isolation and Identification of MTB

Culture is the gold standard diagnostic tool for TB infection in which the causative agent can be isolated and identified as well as used for antimicrobial susceptibility testing. Culture method usually takes longer time about 3 to 4 weeks to show visible colonies on L-J or Ogawa media. However, detection of MTB must be accomplished as rapidly as possible before treatment can be initiated and also to avoid transmission of MTB to other people. As a result, several newer culture methods have been proposed which shorten the time to get the results including radiometric procedures such as BACTEC 460 TB system and fluorescent based detection such as BACTEC MGIT 960 system. Methods using DNA probes are also available to detect specific

genetic markers for identification of mycobacteria isolated in the culture (Ryan *et al.*, 2010; Talaro and Chess, 2011).

2.4.3 Tuberculin Skin Test

This test also called Mantoux test which has been used to measure the DTH. The test uses purified protein derivative (PPD), a standardized solution of TB protein preparation derived from culture fluids of MTB. A positive Mantoux test denotes prior antigenic exposure and the development of DTH due to MTB infection at some time. However this test cannot distinguish between active and latent TB infection (Ryan *et al.*, 2010; Talaro and Chess, 2011).

2.4.4 Nucleic Acid Amplification

Nucleic acid amplification is a molecular technique that uses polymerase chain reaction (PCR) to detect MTB in clinical specimens. Commercially available kit with Food and Drug Administration (FDA) approval is the Amplicor *Mycobacterium tuberculosis* test by Roche Diagnostic Systems, Germany. The kit utilizes PCR to detect MTB directly in respiratory specimens. Another commercially available nucleic acid probe is AccuProbe manufactured by GenProbe, USA. The sensitivity of the assay is 95% to 100% (Forbes, 1995; Forbes *et al.*, 2002; Goodwin, 2007). Furthermore, there is a newly developed technology known as loop-mediated isothermal amplification (LAMP) which is more simple, rapid and sensitive (Iwamoto *et al.*, 2003). Overall, nucleic acid amplification is rapid compared to other method such as culture method (Moore *et al.*, 2005).

2.4.5 Immunodiagnostic Tests

The serological test is another type of immunodiagnostic technique. A test kit called Quantiferon-TB Gold kit was developed (Cellestis Limited, Australia). The test measures the cell-mediated immune response in blood samples to mycobacterial antigens which is then measured using Quantiferon-TB Gold ELISA technique. The technique is more effective due to less affected by BCG vaccination and cross-reactivity with other antigens (Goodwin, 2007).

2.5 Treatment by Anti-TB Drugs

Anti-TB drugs generally kill the bacilli in the lungs, organs as well as macrophages. There are several anti-TB drugs for treating TB patients. The primary drug of choice also called first-line anti-TB drugs which include isoniazid (INH), rifampicin (RMP), ethambutol (EMB), streptomycin (SM) and pyrazinamide (PZA) are used in combination to treat the patients infected with susceptible MTB. These drugs have been widely used with long clinical experience, good efficacy and known side effects. Besides that, there are also second-line anti-TB drugs which less preferred and act as back up or reserve for use in patients who fail to respond to first-line anti-TB drugs. The drugs include para-Aminosalicylic acid (PAS), ethionamide, cycloserine, fluoroquinolones and kanamycin (Ryan *et al.*, 2010; Talaro and Chess, 2011).

The TB patients are usually treated with multiple drugs consisting of two to four types of drugs. Usually for the new TB patients, combinations of four first-line drugs are empirically administered while waiting for drug susceptibility test (DST) results (Ryan *et al.*, 2010; Talaro and Chess, 2011). In Malaysia, according to Clinical Practice Guideline in Management of Tuberculosis (2012), new pulmonary

TB patients will receive treatment of two months combination of INH, RMP, EMB and PZA followed by four months combination of INH and RMP. Second-line anti-TB drugs are used when treatment failure or resistance detected toward first-line drugs. The patients will be treated according to standard MDR-TB regimen. The regimen contains fluoroquinolone, ethionamide, four second-line anti-TB drugs and PZA in the intensive phase (Malaysia Health Technology Assessment Section, 2012). Table 2.3 summarizes the first-line anti-TB drugs used for TB treatment.

Table 2.3: Summary of the first-line anti-TB drugs used for TB treatment

Drugs	Properties	Admission	Mode of action	Resistant	Side effect
INH	Colourless, water soluble and small molecule	Oral	Lose the MTB acid-fastness	Mutation in *katG* and *inhA* genes	Nervous systems and liver damage
RMP	Semisynthetic product and lipid soluble	Oral	Prevent MTB mRNA synthesis	Mutation in *rpoB* gene	Hypersensitivity and liver damage
EMB	Synthetic compound	Oral	Inhibit formation of MTB cell wall	Mutation in *embCAB* genes	Optic neuritis
SM	Structure composes of amino sugar	IM	Inhibit MTB protein synthesis	Mutation in *rrs* and *rpsL* genes	Nephrotoxicity
PZA	Analog to nicotinamide	Oral	Cause MTB non-specific damage	Mutation in *pncA* gene	Liver toxic

IM, intramuscular injection

Source: Rom and Garay (2004); Donald and Mcllleron (2009)

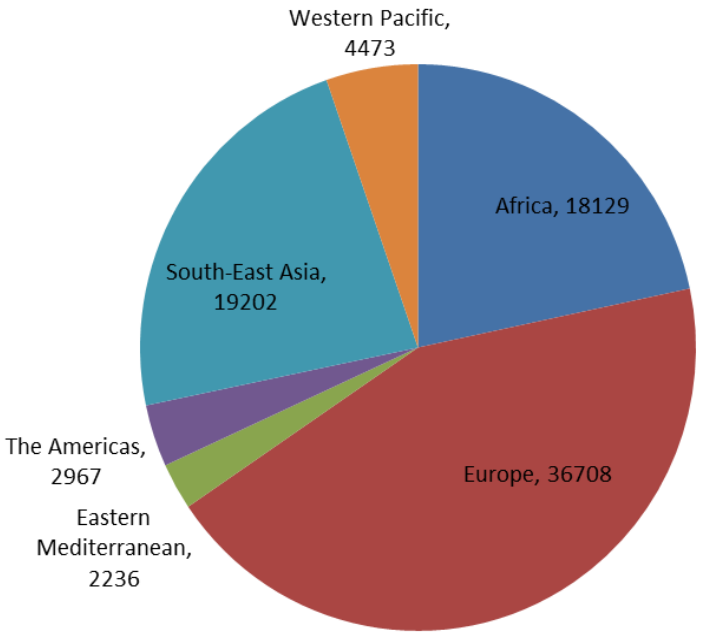
2.6 Drug Resistant Tuberculosis (DR-TB)

2.6.1 Multidrug-Resistant Tuberculosis (MDR-TB)

MDR-TB is caused by MTB strain which is resistant to at least the two most powerful anti-TB drugs, INH and RMP. The emergence of MDR-TB has become a serious problem as a result of global misuse of INH and RMP thus rendering difficulties in the treatment and control of TB (U.S. Department of Health and Human Services, 2012). Global estimation of new TB cases with MDR-TB is 3.5% whereas 20.5% were estimated from previously treated TB cases in 2013 (World Health Organization, 2014). The Eastern Europe and Central Asia had the highest levels of MDR-TB with estimated 450 000 new cases of MDR-TB worldwide and about 300 000 MDR-TB cases were among notified TB patients in 2012 (World Health Organization, 2013).

In Malaysia, a study in the late 80′s reported that the rate of primary resistance to any type of first-line anti-TB drugs was 13 – 15% whereas about 1% was MDR-TB cases (Jalleh *et al.*, 1993). The prevalence of MDR-TB was 0.1% in 1997 (Iyawoo, 2004). The WHO 2013 report stated that total laboratory confirmed MDR-TB cases in Malaysia was 74 cases and the estimated cases of MDR-TB among notified pulmonary TB was 18 cases in 2012 (World Health Organization, 2013). Figure 2.4 shows estimated notified cases of MDR-TB by world region in 2012 and numbers of confirmed MDR-TB cases in Malaysia from 2005 to 2012.

A



B

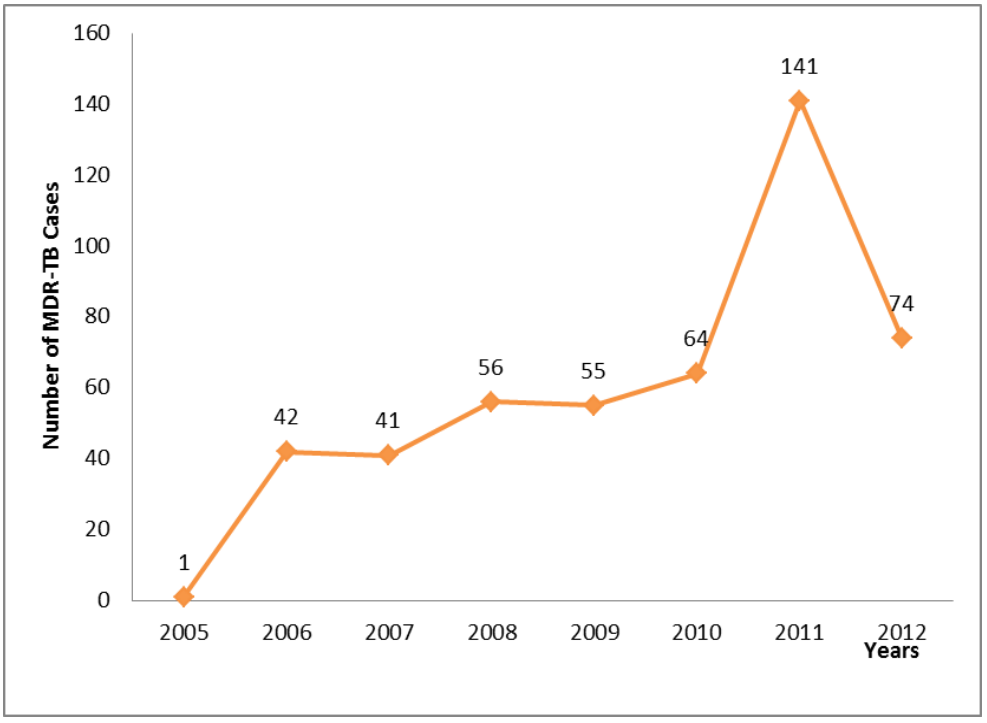


Figure 2.4: A: World region estimated notified cases of MDR-TB in 2012 and B: Number of confirmed cases of MDR-TB in Malaysia, 2005– 2012 [adapted from World Health Organisation (2010) and (2013)]